Dystonin is an essential component of the Schwann cell cytoskeleton at the time of myelination

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SUMMARY

A central role for the Schwann cell cytoskeleton in the process of peripheral nerve myelination has long been suggested. However, there is no genetic or biological evidence as yet to support this assumption. Here we show that dystonia musculorum (dt) mice, which carry mutations in dystonin, a cytoskeletal crosslinker protein, have hypo/amyelinated peripheral nerves. In neonatal dt mice, Schwann cells were arrested at the promyelinating stage and had multiple myelinating lips. Nerve graft experiments and primary cultures of Schwann cells demonstrated that the myelination abnormality in dt mice was autonomous to Schwann cells. In culture, dt Schwann cells showed abnormal polarization and matrix attachment, and had a disorganized cytoskeleton. Finally, we show that the dt mutation was semi-dominant, heterozygous animals presenting hypo- and hyper-myelinated peripheral nerves. Altogether, our results suggest that dt Schwann cells are deficient for basement membrane interaction and demonstrate that dystonin is an essential component of the Schwann cell cytoskeleton at the time of myelination.

Key words: Cytoskeleton, Dystonin/Bpag1, Dystonia musculorum, Schwann cell, Myelination, Mouse

INTRODUCTION

During the process of peripheral nerve myelination, which occurs perinatally in rodents and humans, Schwann cells bundle, ensheath and wrap axons, and they undergo dramatic morphological changes. These changes are mostly dependent on Schwann cell-axon and Schwann cell-basal lamina interactions (Bunge et al., 1982; Obremski et al., 1993), which are thought to be in part mediated by the Schwann cell cytoskeleton. Several studies have addressed the role of the cytoskeleton in peripheral nerve myelination (Trapp et al., 1989, 1995; Kelly et al., 1992; Gillespie et al., 1994; Fernandez-Valle et al., 1997). However, to date there has been no direct genetic, biological or molecular evidence for the importance of the Schwann cell cytoskeleton in this process.

Dystonia musculorum (dt) is a naturally occurring autosomal recessive neuropathy in mice mainly affecting the sensory arm of the nervous system (Duchen et al., 1963; Duchen and Strich, 1964). At 1-2 weeks of age, homozygous dt mice have difficulty in walking, mainly due to their limb incoordination phenotype. Primary and secondary sensory nerve fibers are severely reduced in number and gigantic axonal swellings filled with organelles and neurofilaments are observed as the hallmark of their neurodegeneration (Duchen et al., 1963; Duchen and Strich, 1964; Janota, 1972; Duchen, 1976; Sotelo and Guenet, 1988; Al-Ali and Al-Zuhar, 1989). Surprisingly, nerve fibers of the lower motor neurons appear to be unaffected by the dt mutation, although muscle denervation has been reported in older animals (Duchen, 1976).

We have cloned the dt gene, which we named dystonin (Dst) (Brown et al., 1995). Dst expression during development is predominant in dorsal root ganglia (DRG) and cranial ganglia (Bernier et al., 1995a), which are the main sites of neurodegeneration in dt mice. Dst encodes a neural isoform of bullous pemphigoid antigen 1 (Bpag1) with an additional N-terminal actin binding domain (ABD) (Brown et al., 1995). Bpag1 is a hemidesmosomal protein expressed in squamous epithelia (Jones et al., 1994; Klatte et al., 1989; Mutasim et al., 1985). Bpag1 displays sequence and structural homology with desmoplakin, a desmosomal protein, and with plectin, a large hemidesmosomal protein expressed in many tissues (Green et al., 1992a; Green and Jones, 1996; Elliott et al., 1997). Both of these proteins have been shown to interact directly with intermediate filaments with their C-terminal regions (Green et al., 1992b; Stappenbeck and Green, 1992; Stappenbeck et al., 1993; Wiche et al., 1993; Nikolic et al., 1996). Bpag1 inactivation in mice resulted in dystonia musculorum, fragile skin epithelia and lack of keratin filament association with the inner plate of hemidesmosomes (Guo et al., 1995). Using cell transfection assays, it has been demonstrated that dystonin
(Bpag1n) is able to co-align actin filaments to neurofilaments (Yang et al., 1996). In addition, it has been shown that dystonin N-terminal and C-terminal domains directly bind actin and intermediate filaments, respectively (Yang et al., 1996). The intermediate filament network in dt dorsal root axons is disorganized and, surprisingly, the microtubule network is also severely disorganized (Bernier and Kothary, 1998; Dulpé et al., 1998). Taken together, these studies demonstrate that dystonin is a cytoskeletal crosslinker protein.

The primary target of the dt mutation remains controversial. Most reports have suggested that the axon per se is the main target, since axonal swellings were the first abnormality observed in dt mice (Duchen and Strich, 1964; Janota, 1972; Sotelo and Guenet, 1988). Others have argued that the peripheral nerve degeneration was due to an intrinsic genetic defect of the Schwann cell (Moss, 1981a,b). However, chimeras between wild-type and dt mutant mice demonstrated that the genetic defect leading to sensory axon degeneration was intrinsic to the neuron (Campbell and Peterson, 1992). This latter study, although conclusive, did not exclude the possibility that Schwann cells of dt mice could also be abnormal.

Here we show that in the peripheral nerves of dt mice, two cell types are independently affected: the sensory neuron, which undergoes degeneration, and the Schwann cell, which is defective for myelination. Interestingly, the myelination defect is gene-dosage sensitive, heterozygotes displaying a milder phenotype than homozygous dt mice. We conclude that dystonin is an essential component of the Schwann cell cytoskeleton at the time of myelination.

**MATERIALS AND METHODS**

**Mice**

The dtTg line of mice has been described before (Kothary et al., 1988, 1989) and the spontaneous mouse mutants, dt240 and dt272, were obtained from Jackson Laboratories (Bar Harbor, Maine). Homozygous mutant dt newborn mice were identified by an RFLP analysis as described before (Bernier et al., 1995b).

**Electron microscopy**

Mice were deeply anesthetized by intraperitoneal injection of Avertin, and perfused by cardiac injection of a 37°C solution of 2.5% glutaraldehyde, 2.5% dimethyl sulfoxide and 0.1 M cacodylate (pH 7.2) in PBS. Whole spinal cords including the DRG were dissected out in fixative. The lumbar regions (L1-L6) of the spinal cords were cut out and fixed overnight at 4°C. Samples were post-fixed with osmium tetroxide and mounted vertically in Epon 812. Ultra-thin sections (1 μm) were made transversally and stained with 0.5% Toluidine Blue for light microscopy. Ultrastructural analysis by electron microscopy was made on 60 nm sections after lead citrate-uranyl acetate staining. Identification of dorsal and ventral roots was based on their relative position in the spinal cord and on morphological criteria. Sciatic nerves from the grafting experiment were dissected out in fixative and treated for electron microscopy observation as described above. Note that sections from the middle of the graft were analyzed.

**RNA analysis**

In situ hybridization

Spinal cords from adult mice were fixed in PBS containing 4% paraformaldehyde at 4°C, post-fixed in formalin, embedded in paraffin and processed for in situ hybridization as described previously (Wilkinson and Nieto, 1993; Bernier et al., 1995a; Spector et al., 1998). Expression of the Dst and Plec genes was analyzed by using 0.75 kb and 0.8 kb DNA fragments, respectively, encoding the ABG region. Expression of the Po gene was analyzed by using a 0.65 kb fragment encoding exons 1 to 6. Linearized plasmid was transcribed with RNA polymerase using 35S-labeled ATP or digoxigenin-11-UTP.

**Northern blot analysis**

Total RNA was extracted from sciatic nerves and from brain as described (Chomczynski and Sacchi, 1987). RNA (2-15 μg) was resolved by electrophoresis in agarose/2.2 M formaldehyde, and transferred to nylon membranes (Gene Screen, NEN) using standard techniques (Sambrook et al., 1989). Hybridization with radiolabeled probes was in 50% formamide, 5× SSPE, 5× Denhardt’s, 0.5% SDS and 200 μg/ml salmon sperm DNA for 24 hours at 42°C. Blots were washed at 50°C in several changes of 2× SSC, 0.1% SDS, and autoradiographed at ~80°C.

**RNase protection assays**

Riboprobes labeled with 32P-UTP were generated from linearized plasmid containing the Dst-ABD or Acf7-ABD partial cDNA fragments. These probes allowed us to distinguish between transcripts for isoforms 1 or 2 for each respective gene. The β-actin riboprobe served as a control. Approximately 5×10^4 cpm (Dst or Acf7) and 2.5×10^4 cpm (β-actin) of riboprobe were used per annealing reaction together with 10 μg of total RNA. The total mixture was denatured at 85°C for 5 minutes and annealed for 16 hours at 45°C. RNase digestion was performed at room temperature for 60 minutes using 40 μg/ml of RNase A and 2 μg/ml of ribonuclease T1 (Boehringer-Mannheim). The rest of the procedure was as previously described (Ausubel, 1994).

**RT-PCR**

RT-PCR for Dst and Po was done as described before (Bernier et al., 1996). Annealing temperatures were 55°C (Dst) and 62°C (Po). PCR amplification was for a total of 30 cycles. The Dst primers were 5′-GAGGGCTGTGCTTCGGA TAG-3′ and 5′-CATGTTTGACC- AATGCC-3′ (producing a 736 bp fragment); the Po primers were 5′-CTACCGCTCGCCCGAGGCT-3′ and 5′-GTGCCGGCTGT- GGTCAGCC-3′ (producing a 650 bp fragment).

**Protein analysis**

Proteins were extracted from a pair of sciatic nerves from individual mice directly in protein sample buffer (Laemmli, 1972), separated on a denaturing SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond extra-c, Amersham) by electroblotting (Harlow and Lane, 1988). Membranes were blocked in PBS supplemented with 5% skimmed milk powder. All antibodies were incubated at room temperature in PBS containing 3% BSA. Primary antibodies, the biotinylated secondary antibodies and streptavidin-peroxidase were incubated for 1 hour. Immunoreactive proteins were detected with an enhanced chemiluminescence kit (Amersham, Canada) according to the manufacturer’s instructions. A monoclonal antibody against an extracellular epitope of Po was kindly provided by Dr J. Archelos (University of Würzburg). The antibody against α-tubulin (DM1a) was purchased from Sigma (USA).

**Schwann cell cultures**

Primary cultures of Schwann cells were established using a simplified version of the method described by Cheng and Mudge (1996). Sciatic nerves were dissected from P6-7 (postnatal day 6-7) mice from a dt272/+ x dt272/+ mating. A pair of sciatic nerves from individual mice was placed in an Eppendorf tube containing 1 ml of M2 (HEPES-buffered) embryo culture medium (Hogan et al., 1986). The nerves were enzymatically treated with collagenase (Sigma) and trypsin.
Intrinsic Schwann cell defect in dt mice

(EDTA-free) three times for 15 minutes each at 37°C. Samples were microfuged at 4,000 rpm for 4 minutes, and resuspended in 1 ml of Optimem (Gibco-BRL) supplemented with 10% fetal calf serum. The nerves were gently triturated with a 5 ml syringe sequentially with 18-, 20- and 22-gauge needles. Cells were pelleted by centrifugation at 4,000 rpm for 4 minutes and resuspended in Optimem supplemented with penicillin/streptomycin, 100 mg/l CaCl2 and 1 µM forskolin (Sigma). Cells were plated onto poly-L-lysine- (Sigma) and laminin- (provided by Dr L. McKerracher, Université de Montréal) coated glass coverslips placed in a 24-well tissue culture plate. The medium was replaced daily.

Immunocytochemistry

Cells were fixed for 10 minutes in ice-cold 5% acetic acid in ethanol, washed, and incubated with monoclonal antibody against Po (1:200 dilution) overnight at 4°C. Cells were washed and bound antibodies were detected using a biotinylated anti-mouse Ig and streptavidin-conjugated rhodamine. For tubulin immunofluorescence, cells were fixed in PBS containing 4% paraformaldehyde for 10 minutes at room temperature, washed, permeabilized in PBS containing 0.3% Triton X-100 for 10 minutes at room temperature, washed, and incubated for 1 hour at room temperature with anti-tubulin (DM1a) diluted 1:200 in PBS containing 3% BSA. Cells were washed and bound antibodies were detected using a biotinylated anti-mouse Ig and streptavidin-conjugated rhodamine. F-actin detection was as for tubulin, except that the cells were incubated with rhodamine-phalloidin (Sigma) at 1:200 dilution after the fixation and permeabilization steps.

Sciatic nerve transplants

2-week-old donor mice were killed, their sciatic nerves exposed and removed for transplantation. 6-week-old wild-type recipient mice (CD1 strain) were anesthetized with 2.5% Avertin and the sciatic nerves removed for transplantation. 6-week-old donor mice were allowed to recover and were injected intraperitoneally with the immune suppressant cyclosporin at 25 mg/kg body mass/day for the treatment period. Unaffected animals from the same litters were used as controls. Sensory neurons in the dorsal root degenerate in dt mice, and since this may contribute to the myelination defect, only observations of the ventral root (motor axons) are presented to simplify the study. All of the ventral root axons from a wild-type mouse (a littermate of the dt27J mouse used for Fig. 1F) were well myelinated at this stage and we did not observe any amylaceous axons (Fig. 1A). However, most axons of homozygous dtTg4 (Fig. 1E) and dt27J (Fig. 1F) mice were hypomyelinated. In addition, axonal caliber in the peripheral nerves of dt mice was reduced compared to that in wild-type mice (compare Fig. 1A with 1E,F). The reduction in myelin sheath thickness and in axonal diameter was measured and is summarized in Table 1. The analysis also revealed that a number of large and small caliber axons were amylaceous in dt mice (Table 2). Whether the two dt mutations were non-complementing was addressed. Compound dtTg4/dt27J mice presented the classical dt phenotype and had hypomyelinated ventral root axons (Fig. 1D). This phenotype was almost identical to that of homozygous dtTg4 mice. The percentage of amylaceous axons in the compound heterozygotes was intermediate to dtTg4 and dt27J homozygotes (Table 2). To our surprise, heterozygous dtTg4 and dt27J mice, which do not present any behavioral phenotype or sensory neuron degeneration and are fertile, also had abnormal myelination of their peripheral nerves. In the ventral roots of dtTg4/+ (Fig. 1B) and dt27J/+ (Fig. 1C) mice, some axons appeared hypermyelinated relative to their axonal caliber. In contrast, about 4% of small caliber axons were amylaceous.

### Table 1. Myelin sheath thickness and axonal diameter in ventral roots of P15 dt mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Myelin sheath thickness (µm)*</th>
<th>Axonal diameter (µm)*</th>
<th>Ratio of myelin thickness to axonal diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>4/+ (n=22)</td>
<td>0.96±0.12</td>
<td>4.23±0.61</td>
<td>0.22±0.017</td>
</tr>
<tr>
<td>dtTg4/dtTg4 (n=50)</td>
<td>0.40±0.09</td>
<td>2.76±0.31</td>
<td>0.14±0.032</td>
</tr>
<tr>
<td>dt27J/dt27J (n=45)</td>
<td>0.23±0.16</td>
<td>2.91±0.48</td>
<td>0.079±0.057</td>
</tr>
</tbody>
</table>

*Only large caliber axons that had a myelin sheath were analyzed. Data are means ± s.d. (n = number of values).

### Table 2. Number of amylaceous axons in ventral roots of P15 dt mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of amylaceous large caliber axons/total (%)</th>
<th>Number of amylaceous small caliber axons/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4/+</td>
<td>0/100 (0)</td>
<td>0/23 (0)</td>
</tr>
<tr>
<td>dtTg4/dtTg4 sample 1</td>
<td>10/203 (4.9)</td>
<td>10/51 (19.6)</td>
</tr>
<tr>
<td>dtTg4/dtTg4 sample 2</td>
<td>4/50 (8)</td>
<td>ND</td>
</tr>
<tr>
<td>dt27J/dt27J sample 1</td>
<td>35/71 (49.3)</td>
<td>31/35 (88.5)</td>
</tr>
<tr>
<td>dt27J/dt27J sample 2</td>
<td>44/130 (33.8)</td>
<td>15/23 (65.2)</td>
</tr>
<tr>
<td>dt27J/dt27J sample 1</td>
<td>12/109 (11)</td>
<td>18/37 (48.6)</td>
</tr>
<tr>
<td>dt27J/dt27J sample 2</td>
<td>9/65 (13.8)</td>
<td>9/21 (42.8)</td>
</tr>
</tbody>
</table>

ND, not determined.

Results

Abnormal myelination in homozygous and heterozygous dt mice

Studies were performed on two alleles of dystonia musculorum, dtTg4 and dt27J. The dtTg4 mice have a transgene insertion at the dt locus that is accompanied by a deletion of 45 kb of host genomic sequences (Brown et al., 1994, 1995). This deletion removes two exons from the 5’ end of Dst neural transcripts. The dt27J allele carries an as yet unidentified mutation that leads to aberrant Dst transcripts and reduced Dst transcript levels in the nervous system (Fig. 4E). Both of these dt alleles lack full-length dystonin protein in the nervous system, as judged by immunoblot analysis (Dulpé et al., 1998). At P15, homozygous dtTg4 and dt27J mice displayed the classical dt phenotype. On histological examination, myelination of the CNS appeared normal for both mutant alleles. However, analysis of their peripheral nerves revealed a myelination deficit in both ventral and dorsal roots (not shown). Since it is only the sensory fibers lying in the dorsal root that degenerate in dt mice while motor neurons are unaffected, the presence of severely hypomyelinated fibers in the ventral root was unexpected. Moreover, no gigantic axonal swellings or neurofilament accumulation, which are the hallmarks of neurodegeneration in dt mice, were observed in the motor nerve fibers of these mice. Thus, the myelination defect in dt mice appeared to be independent of the axonopathy.

A systematic ultrastructural analysis of the ventral and dorsal roots at the third lumbar level of homozygous dtTg4 and dt27J P15 mice was performed. Unaffected animals from the same litters were used as controls. Sensory neurons in the dorsal root degenerate in dt mice, and since this may contribute to the myelination defect, only observations of the ventral root (motor axons) are presented to simplify the study. All of the ventral root axons from a wild-type mouse (a littermate of the dt27J mouse used for Fig. 1F) were well myelinated at this stage and we did not observe any amylaceous axons (Fig. 1A). However, most axons of homozygous dtTg4 (Fig. 1E) and dt27J (Fig. 1F) mice were hypomyelinated. In addition, axonal caliber in the peripheral nerves of dt mice was reduced compared to that in wild-type mice (compare Fig. 1A with 1E,F). The reduction in myelin sheath thickness and in axonal diameter was measured and is summarized in Table 1. The analysis also revealed that a number of large and small caliber axons were amylaceous in dt mice (Table 2). Whether the two dt mutations were non-complementing was addressed. Compound dtTg4/dt27J mice presented the classical dt phenotype and had hypomyelinated ventral root axons (Fig. 1D). This phenotype was almost identical to that of homozygous dtTg4 mice. The percentage of amylaceous axons in the compound heterozygotes was intermediate to dtTg4 and dt27J homoyzogotes (Table 2). To our surprise, heterozygous dtTg4 and dt27J mice, which do not present any behavioral phenotype or sensory neuron degeneration and are fertile, also had abnormal myelination of their peripheral nerves. In the ventral roots of dtTg4/+ (Fig. 1B) and dt27J/+ (Fig. 1C) mice, some axons appeared hypermyelinated relative to their axonal caliber. In contrast, about 4% of small caliber axons were amylaceous.
Fig. 1. Myelination defects in *dystonia musculorum* mice. Cross-sections of ventral roots at the lumbar level (L3) from P15 mice observed by electron microscopy. (A) +/- mouse. All axons are myelinated and are of large caliber. (B) *dtTg4/+* mouse and (C) *dt27J/+* mouse. Note the presence of hypo- and hyper-myelinated axons, and a reduction in axonal caliber. Arrows indicate examples of double myelin figures within the same Schwann cell cytoplasm. Inset in C is an example of a hypermyelinated axon. Compare axonal caliber with myelin thickness. (D) Compound *dtTg4/dt27J* mouse; (E) *dtTg4/dtTg4* mouse; and (F) *dt27J/dt27J* mouse. In all three homozygotes there is evidence of hypomyelination, reduction in axonal caliber and amyelinated axons (curved arrows). Bars, 2.2 μm and 1.1 μm for the inset.
Furthermore, axonal caliber was reduced compared to that in wild-type mice. In heterozygous mice of both dt alleles, the presence of double myelin figures within the same Schwann cell cytoplasm was frequently observed (Fig. 1B,C).

**Dysmyelination in neonatal dt mice**

The peripheral nerves at the lumbar level of neonatal homozygous dt27J mice and their wild-type littermates were analyzed by electron microscopy. At embryonic day 18.5, mutant fetuses did not present any myelin figures in either ventral or dorsal roots (not shown). In comparison, many large caliber axons in both roots of wild-type fetuses already showed a thin compact myelin sheath. At P3, when myelination is more advanced, most of the ventral root large caliber axons of wild-type mice already had a robust myelin sheath (Fig. 2A). When compared to wild type, homozygous mutants (Fig. 2B) were devoid of myelinated axons (less than 1%). When myelin was present, unusual myelin figures around the axons or myelin accumulation in the Schwann cell cytoplasm were frequently observed (Fig. 2C). In the mutant, most of the Schwann cells appeared to have arrested after one and half turns around the axon and many of them displayed an abnormal morphology, the inner and outer lips being duplicated (Fig. 2D,E). Finally, the number of Schwann cell nuclei per cross-section of an entire root (P15) or per field (P3) in the ventral roots were counted. At P15, for the dt27J allele, an average of 115 Schwann cell nuclei (from two independent animals) were present per root compared to an average of 26 (from three independent animals) in wild type. This represents a four- to fivefold increase. In contrast, there was no significant difference in the total number of axons per root in dt27J versus wild type (average of 695 versus 726). At P3, the number of Schwann cell nuclei were similar in mutant and wild-type mice.

**Protein zero (Po) protein and mRNA levels are affected in dt mice**

To determine whether the myelination defects in dt mice observed at the ultrastructural level were accompanied by molecular changes, we assessed the levels of specific proteins and transcripts in sciatic nerves. Coomassie Blue staining of an SDS-polyacrylamide gel loaded with sciatic nerve extracts from affected dt27J mice and unaffected littermates is shown in Fig. 3A. The molecular composition of the mutant sciatic nerve extract was modified for many low molecular mass proteins under 40 kDa, which are most of the major constituents of the myelin sheath in peripheral nerves (Po, 32 kDa; Pmp22, 22 kDa; MBP, 18 kDa). Western blot analysis of the sciatic nerve extracts from three different alleles of dt at P15 was performed to assess the level of the major protein of the peripheral nerve, protein zero (Po). As an internal control, a monoclonal antibody against α-tubulin, was used. All three dt alleles presented a reduction in the amount of protein Po relative to tubulin, when compared to an unaffected littermate of each dt allele (Fig. 3B). There was a good correlation between the total amount of Po in the sciatic nerve extracts (compare dt27J with dt3a/- Po levels) and the severity of the myelination deficit observed upon histology, as shown in Fig. 1. This is consistent with the fact that any time there is a reduction in myelin membrane there will be a corresponding reduction in myelin transmembrane protein levels. The additional mutant analyzed on the western blot, dt26J, is another independent dt allele in which full-length dystonin protein is absent in the nervous system (Dalpé et al., 1998). Histological analysis showed that this allele also suffered from a hypomyelination phenotype that is more severe than the dt3a allele (not shown).

Whether mRNA levels for the major myelin genes were perturbed in dt mice was investigated. Total RNA extracts of sciatic nerves from individual P3 homozygous dt16d and dt27J, compound dt16d/dt27J, heterozygous dt16d and dt27J, and wild-type mice were analyzed on northern blots. At this age, homozygous dt mice are phenotypically indistinguishable from their normal littermates. Our results showed that Po mRNA levels were reduced approximately two- to threefold in homozygous dt mice compared to wild-type mice (Fig. 3C). The same mRNA
reduction was observed for \( Pmp22 \). Curiously, heterozygous \( dt^{27J} \) mice presented an approximately twofold increase in both \( Po \) and \( Pmp22 \) mRNA levels compared to their wild-type littermates (Fig. 3C). Heterozygous \( dt^{Tg4} \) mice also showed a small increase in the level of \( Po \) and \( Pmp22 \) transcripts. The signals from this and similar blots were measured on an Instant Imager and the results compiled in Fig. 3D. Mouse \( Gapdh \) mRNA and 28S rRNA levels were used as a control for quantitation.

**\( Dst \) is expressed in Schwann cells**

We have previously reported on the \( Dst \) gene expression pattern during mouse embryogenesis and in the mature CNS (Bernier et al., 1995a). In that study, a 0.2 kb \( Dst \) isoform 2-specific exon was used as a probe for in situ hybridization to demonstrate predominant expression of \( Dst \) in cranial and spinal nerve ganglia. Here a larger riboprobe of 0.75 kb, which encompasses exons encoding the ABD of \( Dst \), was used. On sections of adult spinal cord, strong hybridization was observed in the DRG and in the gray matter, including the lower motor neurons, and in the spinal nerves (Fig. 4B-D). Some hybridization was observed in the white matter as well (Fig. 4B). \( Dst \) expression on single peripheral nerve fibers was observed. The signal was periodic and appeared to coincide with Schwann cell nuclei present along the length of the nerve fiber (Fig. 4C,D). To confirm that the \( Dst \) expression observed was in fact in Schwann cells, similar sections were hybridized with \( Po \), an independent marker of Schwann cells. As seen in Fig. 4D, both \( Dst \) and \( Po \) hybridized to Schwann cells present in the spinal root. By comparison, plectin (\( Plec \)) expression was restricted to the DRG and to some lower motor neurons in the spinal cord, and was not detected in Schwann cells (Fig. 4A).
RNase protection assays on RNA extracted from adult mouse tissues (spinal cords devoid of DRG, DRG and sciatic nerves) were also performed. Using a Dst riboprobe that allowed us to distinguish between Dst isoforms 1 and 2, a strong protection of a large fragment (Dst isoform 1, upper band) and of a smaller fragment (Dst isoform 2, lower band) in both spinal cord and DRG extracts was observed (Fig. 4E). Some hybridization was also observed in the white matter. (C) High magnification view of the DRG and spinal nerve from the section shown in B. Hybridization to nerve fibers is in a periodic manner (arrows). Bar, 193 μm. (D) High magnification views of nerve fibers hybridized with either Dst or Po digoxigenin-labeled riboprobes. Po served as a marker of Schwann cells. Both Dst and Po gave similar results, hybridizing to Schwann cells located in a periodic manner along the length of individual nerve fibers (arrows). Magnification, 400x. (E) RNase protection analysis of total RNA isolated from spinal cords, DRG and sciatic nerves. Riboprobes were either Acf7 (left panel) or Dst (right panel). The probes were designed to allow us to distinguish between transcripts encoding isoforms 1 (Iso-I) and 2 (Iso-II). Mouse β-actin riboprobe was included as a control for quantitation. Note that Dst transcripts are more readily detected in sciatic nerve RNA than are Acf7 transcripts.

RNase protection assays on RNA extracted from adult mouse tissues (spinal cords devoid of DRG, DRG and sciatic nerves) were also performed. Using a Dst riboprobe that allowed us to distinguish between Dst isoforms 1 and 2, a strong protection of a large fragment (Dst isoform 1, upper band) and of a smaller fragment (Dst isoform 2, lower band) in both spinal cord and DRG extracts was observed (Fig. 4E). Note that Dst isoform 2 is more abundant than isoform 1 in these tissues (3:1 ratio). Consistent with the in situ hybridization results, protected bands in the sciatic nerve extract were also observed (Fig. 4E). In sciatic nerve, the ratio between Dst isoforms 1 and 2 signals was now inversed compared to that in spinal cord and DRG extracts. As control, RNA from homozygous dt27J and dtTg4 spinal cord extracts (P15) were used. In the dt27J extract, the protected bands were reduced in amount compared to the normal mouse by three-to fourfold (Fig. 4E), consistent with northern blot analysis that showed reduced levels and aberrant Dst transcripts in spinal cord RNA extracts from this mutant allele (not shown). In RNA extracts from dtTg4 mice, no protected bands were observed (Fig. 4E). This latter result was expected since dtTg4 mice have a large genomic deletion that removes exons A and A' from the dystonin gene (Brown et al., 1995). This alteration causes a dramatic reduction in Dst transcription downstream of the deletion (G. B. and R. K., unpublished). As a comparison, a riboprobe from the ABD encoding region of the mouse Acf7 cDNA (Bernier et al., 1996) was used. Acf7 is another putative cytoskeletal crosslinker protein with strong similarities to plectin and dystonin. In particular, the ABD region of Acf7 displays 76% identity at the amino acid level with that of dystonin (Bernier et al., 1996). RNase protection showed that Acf7 was expressed in the spinal cord, DRG and sciatic nerve, but at much lower levels then Dst (Fig. 4E).
RT-PCR analysis was done to further demonstrate \textit{Dst} expression in Schwann cells. Primers specific for \textit{Dst} amplified the correct fragment from RNA of brain, sciatic nerve and primary culture of Schwann cells (Fig. 5A). By comparison, primers specific for \textit{Po} amplified the right fragment in sciatic nerve and primary Schwann cell culture (Fig. 5A). Finally, the size of the \textit{Dst} transcript in Schwann cells was assessed by analyzing the RNA extracted from newborn mouse sciatic nerves on northern blots. Using cDNA sequences encoding exons 2 and 3 of \textit{Bpag1} as a probe, a \textit{Dst} transcript of approx. 15 kb was observed in sciatic nerve RNA, which is identical in size to transcript seen in brain (Fig. 5B). Note that there was no evidence for the presence of the 9 kb \textit{Bpag1} transcript. All these results are in agreement with the previous expression study on \textit{Dst} (Bernier et al., 1995a). In addition, they now provide strong evidence that \textit{Dst} is expressed in Schwann cells.

\textbf{Cultured \textit{dt} Schwann cells have abnormal morphology and appear deficient for substrate attachment}

Primary cultures of Schwann cells from the sciatic nerves of 6- to 7-day-old pups from \textit{dt} \textit{27J/}+ \textit{dt} \textit{27J/}+ crosses were established. After 1 day in culture, wild-type (Fig. 6A) or heterozygous \textit{dt} \textit{27J} (not shown) Schwann cells were bipolar and had long cytoplasmic elongations. At this stage, the cells were not organized and numerous fibroblasts were present. After 4 days in culture, less than 5\% of the cells were fibroblasts, and Schwann cells aligned in an ordered fashion. This is apparent in Fig. 6C, where cells were labeled with a tubulin antibody. In contrast, Schwann cells from sciatic nerves of homozygous \textit{dt} \textit{27J} mice were round and refractile after 1 day in culture (Fig. 6B). Some had a bipolar morphology but with very short and thin cytoplasmic extensions. This was not caused by a cell density phenomenon, since similar numbers of Schwann cells were present in both cultures. When labeled for tubulin at day 4, mutant Schwann cells presented an abnormal morphology and had not aligned (Fig. 6D). To confirm that the cultures contained Schwann cells, an antibody against protein \textit{Po} was used. In wild-type \textit{dt} cultures at day 4, almost all the cells presented a granular cytoplasmic labeling with this antibody (Fig. 6E). In addition, some cells contained enormous perinuclear cytoplasmic vesicles that were strongly labeled. In these cells, the nuclei were displaced at the periphery. These observations show that a population of Schwann cells in the cultures overexpress the protein \textit{Po}. In the mutant culture, most of the cells also showed a granular cytoplasmic labeling (Fig. 6F). However, very few, if any of these cells presented strong \textit{Po} labeling in vesicle-like structures, as described for the wild-type cultures.

In wild-type (Fig. 7A) and \textit{dt} heterozygous Schwann cells (not shown), microtubules radiated from the nucleus to the periphery. In the homozygous \textit{dt} \textit{27J} Schwann cells, microtubule network organization appeared dense and perturbed (Fig. 7B). Using rhodamine-phalloidin, filamentous actin network organization in the cultures was also analyzed. In wild-type (Fig. 7C) and heterozygous \textit{dt} Schwann cells (not shown), F-actin labeling was concentrated in small peripheral cytoplasmic extensions that appeared to be attached to the coverslip surface. Almost all of the Schwann cells were labeled in this manner. In mutant Schwann cells, F-actin labeling was very weak in most of the cells. However, some cells presented a very strong labeling. In these round-shaped cells, numerous long and thin cytoplasmic extensions radiating from the same cell were labeled with phalloidin (Fig. 7D).

Finally, similar experiments were performed on pups from \textit{dt} \textit{108y/}+ \times \textit{dt} \textit{108y/}+ crosses. Cultured homozygous \textit{dt} \textit{108y} Schwann cells also displayed morphological abnormalities, albeit milder than those of \textit{dt} \textit{27J} mutant Schwann cells (not shown).

\textbf{Schwann cell abnormality in \textit{dt} mice is cell autonomous}

The myelination defect in \textit{dt} mice could either be intrinsic to axons or to Schwann cells, or both. To address this question, a portion of the sciatic nerve from ++, \textit{dt} \textit{27J/+}, \textit{dt} \textit{27J/dt} \textit{27J} and \textit{dt} \textit{108y/dt} \textit{108y} mice \((n = 2, 1, 2 \text{ and } 2, \text{ respectively})\) was transplanted to the sciatic nerve of a wild-type recipient mouse. This allowed us to observe in vivo myelination of the host regenerating axon by the transplanted Schwann cells. Using \textit{dt/dt} mice as recipients was not possible because all the \textit{dt} mice in our colony die between the second and fourth week after birth. Histological analysis of the wild-type graft after 1 month showed numerous well-myelinated axons of all calibers (Fig. 6).
Intrinsic Schwann cell defect in dt mice

Myelination was almost comparable in the dt27J/+ graft (Fig. 8B). However, in this graft, hypomyelinated axons and numerous gigantic hypermyelinated axons (tomacula) were also observed (Figs 8B, 9A). In the homozygous dtTg4 graft (Fig. 8C), axons were hypomyelinated, although not severely, compared to the wild-type graft. Finally, in the homozygous...
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Fig. 8. The myelination defect in dt mice is intrinsic to Schwann cells. Nerve grafting experiments. A portion of the sciatic nerve from +/+ (A), dt27/+ (B), dtTg4/dtTg4 (C) and dt29/dt27 (D) mice was transplanted to the sciatic nerve of wild-type recipient mice. 1 month post-transplantation, the grafts were recovered and processed for electron microscope observation. Sections were from the middle of the graft. An example of a hypermyelinated axon is shown (B, arrow). Bar, 2 µm.

dt29 graft (Fig. 8D), axons were severely hypomyelinated compared to the wild-type graft.

To our surprise, the control dt27/+ graft presented numerous tomacula. These were not observed in the peripheral nerves of 2-week-old dt27/+ and dtTg4/+ animals, although some axons appeared hypermyelinated (Fig. 1). However, histological examination of the sciatic nerve of an 8-month-old dt27/+ animal (Fig. 9B) revealed the presence of tomacula (about five per whole sciatic nerve cross section). Thus, the nerve graft experiment has reproduced, although more drastically, the myelination abnormality observed in dt27/+ mice. Finally, in the homozygous dt27/+ graft, multiple cases of premature demyelination were observed, as suggested by the presence of a basement membrane around naked axonal membranes (Fig. 9D). As well, axonal death probably occurred in this graft, as indicated by the significant reduction in axonal density in the graft and the presence of axon ‘ghosts’ (Fig. 9C). Altogether, the graft experiments strongly suggest that the Schwann cell abnormality in dt mice is cell-autonomous.

DISCUSSION

Two independent cell types are targeted by the dt mutation in peripheral nerves

Our study now provides strong evidence that two cell types are independently affected in the peripheral nerves of dt mice. We have demonstrated delayed, reduced and aberrant myelination in the peripheral nerves of homozygous dt mice. Moreover, heterozygous dt mice, which were up to now believed to be completely normal, also present myelination abnormalities. The myelination defects observed in vivo were intrinsic to the Schwann cells and were almost entirely reproduced in cell culture and nerve graft experiments. Finally, we have demonstrated that Dst, the gene defective in dt mice, is
expressed in both motor and sensory neurons, as well as in Schwann cells.

It is noteworthy that axonal caliber is reduced in both homozygous and heterozygous dt mice. In our experiments where sciatic nerve segments from homozygous dt mice were transplanted to wild-type recipients, regenerating fibers also had reduced axonal caliber compared to wild-type grafts. However, the graft of the sciatic nerve from a dt27J/+ mouse did not display this phenomenon. Since Dst is expressed in motor neurons, axonal caliber reduction might be due in part to an intrinsic defect of the neuron. On the other hand, myelination has been shown to have profound effects on axonal caliber (de Waegh et al., 1992). This leaves open the possibility that reduction in axonal caliber in dt/dt and dt/+ mice could be due to the Schwann cell defect and/or that dystonin has a function in neurons that is essential for radial axonal growth.

**Schwann cell defect in dt mice is gene-dosage sensitive**

dt mice present a myelination defect that is Schwann cell-dependent. Homozygous dt^{g4} mice have hypomyelinated axons with some rare amylinated axons. Homozygous dt^{27} mice also have hypomyelinated axons but with abundant amylinated axons (dysmyelination). As well, heterozygous dt mice present myelination abnormalities that are less severe than those in homozygous dt mice. Thus, the myelination defect in dt mice is gene-dosage sensitive, or semi-dominant. The reasons for the difference in severity of phenotype between these two dt alleles are not clear. However, based on our observations of an intermediate phenotype in the compound heterozygotes (Table 2), it is possible that the dt^{g4} allele is a hypomorphic allele. Genetic background variation between these dt alleles is another possible explanation for the difference in severity of phenotype. As well, it is known that different mutations within the same gene can lead to highly variable phenotypes (Bedell et al., 1997 and references therein).

Interestingly, allelic variation and gene-dosage sensitivity are both common phenomena in peripheral neuropathies involving myelination. In heterozygous mutant trembler mice, which carry point mutations in the Pmp22 gene (Suter et al., 1992a,b), the phenotype is more severe compared to heterozygous Pmp22 knock-out mice (Adlkofer et al., 1995). As well, in patients carrying mutations in the myelin protein zero (MPZ) gene, different mutations lead to neuropathies with varying degrees of severity (Warner et al., 1996). In mice with an ablated Po gene (Giese et al., 1992; Martini et al., 1995), peripheral nerves are severely hypomyelinated, and myelin and axons degenerate. In Po/+ animals, myelination is normal at first, but older animals (4 months) develop progressive demyelination. In Pmp22 knock-out mice (Adlkofer et al., 1995), homozygous mutant animals are retarded in their onset of myelination, but later develop hypermyelinated structures (tomacula) at a young age that lead to demyelination. Pmp22/+ animals present normal myelination at first, but later develop focal tomacula as well. Finally, in transgenic rats that overexpress the Pmp22 gene (Sereda et al., 1996), hypomyelination and onion bulb formation (single transgenic) and amyelination (double transgenic) are observed.

In dt mice, both Po and Pmp22 genes are underexpressed at the RNA level in 3-day-old homozygous animals. In heterozygous animals, these genes are overexpressed, and more dramatically so in the dt^{27} allele (approximately twofold). Curiously, this seems to correlate with the severity of the phenotype of each mouse. Homozygous dt^{27} mice display a more severe hypomyelination phenotype than the dt^{g4} mice, and this correlates with a more severe reduction in Po and Pmp22 gene expression. The dt^{27} heterozygous mice overexpress Po and Pmp22, and present what could be interpreted as premature myelination. They also present...
heterodimeric proteins involved in cell interactions with the extracellular matrix and are the receptors for laminin, fibronectin and other basement membrane proteins (Tryggvason, 1993). Integrin cytoplasmic domains are thought to interact with the actin- or intermediate filament-based cytoskeleton, allowing intracellular signaling from the basement membrane to the cytoskeleton (Hynes, 1992). Integrin α6β4 is present in the hemidesmosomes of epithelial cells (Stepp et al., 1990), as well as in Schwann cells. Expression studies and co-culture assays have suggested a role for integrin β4 in peripheral nerve myelination (Einheber et al., 1993; Feltli et al., 1994). Gene targeting of integrin β4 in mice resulted in severe skin blistering and early postnatal death (Dowling et al., 1996; van der Neut et al., 1996). No reports as yet clearly address whether myelination is affected in these mice. However, the dy mice, which carry a mutation in the laminin α2 gene (Lama2), have severely hypomyelinated peripheral nerves due to a basement membrane defect (Madrid et al., 1975; Sunada et al., 1994; Xu et al., 1994). All these observations suggest that a molecular cascade exists that mediates signaling from the basement membrane to the cytoskeleton in Schwann cells at the time of myelination. How and where dystonin would be involved in this cascade is not known. A recent report has demonstrated that the cytoplasmic tail of integrin β4 interacts directly or indirectly with HD1 (hemidesmosomal protein 1; Hieda et al., 1992) in Cos cells (Niessen et al., 1997). However, the real molecular nature of HD1 is not known, although it has been speculated that it might be plectin. In our study, plectin expression at the RNA level was negligible in Schwann cells. In addition, a myelination defect in the peripheral nerves of humans carrying homozygous mutations in the PLEC gene has not been reported (Chavanas et al., 1996; McLean et al., 1996; Pulkkinen et al., 1996; Smith et al., 1996). A targeted mutation in the mouse Plec gene resulted in loss of skin and muscle cytoarchitectural integrity (Andrä et al., 1997). These mice die 2-3 days after birth, and myelination defects were not reported. In any case, in Schwann cells, the integrin β4 cytoplasmic tail might be able to interact with high molecular mass proteins involved in actin and intermediate filament cytoskeleton organization, such as HD1, plectin or dystonin. Considering the genetic, molecular and biological evidence presented here, as well as the cytoskeleton perturbation observed in our dt Schwann cell cultures, it is tempting to speculate that dystonin is in fact playing this role in Schwann cells.

Other mechanisms for how dystonin is implicated in myelination are possible and need not be mutually exclusive. For example, actin filaments might be involved in the progression of the inner lip and the generation of the mechanical force during myelination. Dystonin is able to link actin filaments (Yang et al., 1996), and actin organization is perturbed in dt mutant Schwann cells in culture. As well, dystonin is able to link actin filaments to intermediate filaments (Yang et al., 1996), and thus might be involved in the integration of the actin and intermediate filament cytoskeleton during the process of inner lip progression. Finally, microfilament and intermediate filament perturbation through dystonin mutation might result in microtubule disorganization, and thus lead to vesicle transport defects. Considering the high metabolic activity of the Schwann cell at the time of myelination, this would be predicted to impair normal myelination.

**Molecular mechanism of the myelination defect in dt mice**

Dystonin is a cytoskeleton organizer protein in the nervous system, linking microfilaments to intermediate filaments (Brown et al., 1995; Guo et al., 1995; Yang et al., 1996). We show here that Dst is expressed in Schwann cells, and that Dst mutations result in myelination defects that are intrinsic to Schwann cells. In homozygous dt mice, basement membrane production occurs, but Schwann cells temporarily arrest at the promyelinating stage, and produce supernumerary outer and inner lips. The unusual morphology suggests that mutant Schwann cells may have polarization problems in vivo. This observation is interesting since it has been proposed that basement membrane-Schwann cell interaction is essential to generate polarization of the Schwann cell cytoplasm after the ensheathment stage, to allow progression through the wrapping stage (Bunge et al., 1989). This leads to the generation of an asymmetric movement around the axon by the Schwann cell, the Schwann cell outer lip being blocked by its contact with the basement membrane and the inner lip being allowed to progress by its contact with the axon and the apposing Schwann cell membrane. Thus, it is possible that the Schwann cell defect in dt mice is linked to a loss of contact inhibition with the basement membrane, leading to an absence of Schwann cell polarization. In our culture experiments, mutant Schwann cells tended to be multipolar. In addition, these cells remained refractile and produced very short cytoplasmic extensions, suggesting that they might be deficient for proper attachment or interaction with the poly-L-lysine/laminin substratum. Interestingly, F-actin is known to be involved in focal adhesion (reviewed in Burridge et al., 1990) and F-actin attachment or interaction with the poly-L-lysine/laminin substrate. Interestingly, F-actin is known to be involved in focal adhesion (reviewed in Burridge et al., 1990) and F-actin staining was dramatically reduced or aberrant in the cultured mutant Schwann cells, leaving open the possibility that focal adhesion may be abnormal in these cells.

In heterozygous dt mice, Schwann cells show hypo- and hyper-myelination, supernumerary myelin figures, and produce late hypermyelinated axons (tomacula). Again, these phenomena could be interpreted as a reduction in contact inhibition with the basement membrane. One gene dosage is sufficient to promote Schwann cell polarization (as seen in the Schwann cell culture experiments); however, myelination speed would be increased due to a reduced signal inhibition from the basement membrane. This is reflected by the overexpression of the myelin genes, Po and Pmp22. Asynchrony between myelination and radial axonal growth would result in either hypo- or hyper-myelinated axons. As well, lack of contact inhibition with basement membranes at the paranode would lead to the late tomacula, since tomacula usually appear at these sites (Adlkofer et al., 1995).

Interestingly, phenomena such as cell polarization, basement membrane interaction, matrix attachment or focal adhesion are all thought to be mostly mediated by integrins (for reviews see Hynes, 1992; Fäsself et al., 1996). Integrins are transmembrane heterodimeric proteins involved in cell interactions with the Schwann cell and thus lead to vesicle transport defects. Considering the high metabolic activity of the Schwann cell at the time of myelination, this would be predicted to impair normal myelination.
In conclusion, the search for Schwann cell cytoskeletal proteins that are essential for myelination has been the focus of recent research. Cloning of periaxin (Gillespie et al., 1994) and characterization of intermediate filaments expressed in Schwann cells (Kelly et al., 1992) are some examples of the progress made. We have presented evidence that mutations in a cytoskeletal protein expressed in Schwann cells, dystonin, leads to a myelination defect that is gene-dosage sensitive and intrinsic to Schwann cells.

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